

Purification and Characterization of Oocyte Cytoplasmic Tubulin and Meiotic Spindle Tubulin of the Surf Clam *Spisula solidissima*

KATHY A. SUPRENANT and LIONEL I. REBHUN

Biology Department, University of Virginia, Charlottesville, Virginia 22901; and Marine Biological Laboratory, Woods Hole, Massachusetts 02543

ABSTRACT Assembly-competent tubulin was purified from the cytoplasm of unfertilized and parthenogenetically activated oocytes, and from isolated meiotic spindles of the surf clam, *Spisula solidissima*. At 22°C or 37°C, *Spisula* tubulin assembled into 48–51-nm microtubules during the first cycle of polymerization and 25-nm microtubules during the third and subsequent cycles of assembly. Microtubules were formed from sheets of 26–27 protofilaments helically arranged at a 36° angle relative to the long axis of the polymer and were composed of α and β tubulins and several other proteins ranging in molecular weight from 30,000 to 270,000. Third cycle microtubules contained 14–15 protofilaments in cross-section and were composed of >95% α and β tubulins. After three cycles of polymerization at 37°C, unfertilized and activated oocyte tubulin self-assembled into microtubules at a critical concentration (Ccr) of 0.09 mg/ml. At the physiological temperature of 22°C, unfertilized oocyte tubulin assembled into microtubules at a Ccr of 0.36 mg/ml, activated oocyte tubulin assembled at a Ccr of 0.42 mg/ml, and isolated meiotic spindle tubulin assembled at a Ccr of 0.33 mg/ml. The isoelectric points of tubulin from both unfertilized oocytes and isolated meiotic spindles were 5.8 for α tubulin and 5.6 for β tubulin. In addition, one dimensional peptide maps of oocyte and spindle α and β tubulins were very similar, if not identical. These results indicate that unfertilized oocyte tubulin and tubulin isolated from the first meiotic spindle are indistinguishable on the basis of assembly properties, isoelectric focusing, and one dimensional peptide mapping. These results suggest that the transition of tubulin from the quiescent oocyte state to that competent to form spindle microtubules in vivo does not require special modification of tubulin but may involve changes in the availability of microtubule organizing centers or assembly-promoting microtubule-associated proteins.

In 1972, Weisenberg (68, 69) described a particulate and sedimentable pool of tubulin that was assayed indirectly by colchicine binding in homogenates of unfertilized surf clam oocytes. The sedimentable tubulin was present in unfertilized oocytes in a granular and filamentous matrix suggested to be a storage form of tubulin. The level of sedimentable tubulin decreased to a minimum level at 5 min after parthenogenetic activation (time of nuclear envelope breakdown) and rose to a maximum value at meiotic metaphase. In addition to sedimentable tubulin, Burnside et al. (10) demonstrated using vinblastine precipitation, that surf clam oocytes contained a large pool of soluble tubulin that was maintained at a constant level throughout early embryogenesis. Further, Weisenberg

and Rosenfeld (70) showed that homogenates of activated *Spisula solidissima* oocytes at 28°C would assemble into microtubules, forming aster-like structures associated with centrioles. No microtubule assembly or aster formation was observed when homogenates of unfertilized oocytes were warmed, although a crude preparation of organizing centers from activated oocytes induced aster formation in homogenates of unactivated oocytes. Unfortunately, the complexity of the fractions used did not allow a critical analysis of the factors or lack thereof, that may be necessary for microtubule assembly in *Spisula* oocytes. For example, the activated microtubule-organizing center preparation may contain factors that modify the tubulin prior to its assembly. Thus, unfertil-

ized oocyte tubulin may be incompetent to assemble until fertilization or activation, at which time the tubulin becomes chemically modified to a polymerization-competent form. Alternatively, the tubulin may be competent to polymerize but is prevented from doing so by compartmentalization of the tubulin or the presence of an inhibitor of microtubule assembly (3, 9, 42, 72). In addition, fertilization may trigger the release or synthesis or microtubule-associated proteins (MAPs)¹ (15, 29, 39, 53) or microtubule organizing centers (MTOCs) (46) necessary for microtubule assembly in these oocytes.

To initiate studies of these problems, we have compared tubulin from the unfertilized oocyte, activated oocyte, and first meiotic spindle apparatus of the surf clam, *Spisula solidissima*, by in vitro assembly at physiological temperatures, isoelectric focusing, and one dimensional peptide mapping. In addition, we have described the assembly of surf clam tubulin into 26–27 protofilament microtubules during the first cycle of in vitro assembly and 14–15 protofilament microtubules during the third and subsequent cycles of assembly. In all properties examined, we find little difference between tubulins isolated from these various stages of development, suggesting that fertilization or activation does not change the biochemical properties of oocyte tubulin. Parts of this work have appeared previously in abstract form (56).

MATERIALS AND METHODS

Préparation of Oocytes: Ovaries were dissected from ripe surf clams (*Spisula solidissima*, collected during the summer months at the Marine Biological Laboratory, Woods Hole, MA), minced in filtered natural sea water, and then filtered through cheesecloth. The oocytes were washed three times by settling through fresh filtered sea water. To remove sea salts that might interfere in the isolation of tubulin and to remove the vitelline membrane, oocytes were washed twice in 1 M glycerol containing 10 mM sodium phosphate, pH 7.8, and then washed once with 1 M glycerol alone (25). The oocytes were harvested and packed with a hand cranked centrifuge.

For some experiments, surf clam oocytes were parthenogenetically activated to initiate meiosis by the addition of 14 ml of 0.5 M KCl in distilled water to a dilute suspension of oocytes (5 ml in 86 ml sea water) (1). The first meiotic metaphase was reached in 12–13 min (at 22°C) after activation with KCl. Activated oocytes were then washed with buffered glycerol (see above) and harvested by centrifugation.

Isolation of Meiotic Spindles from Activated Oocytes: Hexylene glycol (2-methyl-2,4-pentanediol) augmented meiotic spindles (48) were isolated as described by Keller and Rebhun (25). Unfertilized surf clam oocytes were activated with KCl and were allowed to develop to early metaphase of the first meiotic division. Activation of oocytes was monitored by examining the development of birefringent spindle fibers with polarization light microscopy. Activated oocytes were washed once in buffered glycerol and resuspended in 1 M glycerol containing 3% hexylene glycol (vol/vol) for 3 min. Hexylene glycol augmented the spindles such that very large, birefringent spindles formed as previously described (48). Spindles were isolated by vortexing the activated oocytes into isolation buffer (modified from Keller and Rebhun, 25) containing 100 mM PIPES/NaOH, pH 6.6, 1 mM MgCl₂, 1 mM EGTA, and 1% Nonidet P-40 (wt/vol). The disrupted cells were centrifuged in a hand cranked centrifuge to pellet cortices and intact oocytes. The meiotic spindles remaining in the supernatant were pelleted by spinning for 2 min in a clinical centrifuge at 2,000 g. The spindles were resuspended in isolation buffer without NP-40 and recentrifuged. This was repeated until a clean spindle preparation was obtained (usually two to three washes).

Purification of Meiotic Spindle Tubulin: Meiotic spindles, isolated as described above, were placed on ice after addition of GTP and dithiothreitol (DTT) to the spindle preparation, at final concentrations of 0.5 and 2 mM, respectively. Spindles were broken up by passage through a 1-ml syringe with a 25-gauge needle and the loss of spindle birefringence was monitored by polarization light microscopy. After 30–40 min at 4°C, the

supernatant containing the depolymerized spindle tubulin was removed (usually 100–200 μ l), made 1 mM in GTP, and warmed to 30°C to assemble tubules. Tubules were pelleted by centrifugation at 40,000 g for 20 min at 30°C and were stored at –80°C until used.

Purification of Total Cytoplasmic Tubulin: Total cytoplasmic tubulin was isolated from unfertilized oocytes and KCl-activated oocytes by DEAE-column chromatography and three cycles of temperature-dependent assembly and disassembly, modified by Suprenant and Rebhun (57) from Kuriyama's original methods (31). After packing with a hand cranked centrifuge, 15–20 ml of unfertilized or KCl-activated oocytes were homogenized at 4°C in an equivalent volume of homogenization buffer (20 mM sodium phosphate, pH 6.8, 1 mM EGTA, 5 mM MgCl₂, 1 M glycerol, and 0.1 mM GTP). The homogenate was centrifuged at 100,000 g for 60 min. The resulting supernatant was made 50% in ammonium sulfate (saturation, 313 g/l) and centrifuged at 27,000 g for 20 min. The ammonium sulfate precipitate was resuspended in column buffer (homogenization buffer without glycerol) and clarified by centrifugation at 12,000 g for 10 min. The supernatant was loaded on a DEAE-Sephadex (A 50–120) column (20–25-ml bed vol) equilibrated in column buffer. The column was washed with 10 column volumes of column buffer containing 0.35 M NaCl. Tubulin was eluted with column buffer containing 0.65 M NaCl at a flow rate of 15–20 ml/h. 0.5-ml fractions were collected and protein elution was monitored by measuring the absorbance of the fractions at 280 nm using column buffer containing 0.35 M NaCl as a reference. Tubulin-containing fractions were concentrated by 50% ammonium sulfate precipitation and were centrifuged at 27,000 g for 20 min.

Tubulin was further purified by cycles of temperature-dependent assembly and disassembly. The ammonium sulfate precipitate was resuspended in 0.3–1.0 ml of assembly buffer (100 mM PIPES/KOH, pH 6.80, 1 mM MgCl₂, 1 mM EGTA, and 1 mM GTP) and was desalted by centrifuge column chromatography (50, 57) using Sephadex G-25 equilibrated in assembly buffer. The desalted tubulin-containing fractions were warmed to 37°C to assemble tubules that were then pelleted at 40,000 g for 20 min at 37°C. These tubules, referred to as first cycle tubules, were stored at –80°C until used.

Brain Microtubule Protein: Bovine brain microtubule protein was obtained by temperature-dependent assembly and disassembly as previously described (44, 52). The initial cycle of microtubule assembly was done in assembly buffer with 3.4 M glycerol. Subsequent cycles of assembly and disassembly were done in assembly buffer without glycerol. Bovine brain MAPs were obtained by phosphocellulose chromatography of twice-cycled microtubule proteins (53, 67).

Sedimentation Assay for Assembly of Tubulin: All microtubule or microtubule assembly experiments were done in assembly buffer (note that spindle isolation buffer is different from assembly buffer). Tubule pellets (first cycle) were resuspended in cold assembly buffer, depolymerized on ice for 30 min, and centrifuged at 100,000 g for 20 min to remove denatured proteins and nonmicrotubule-associated proteins. The soluble protein in the cold supernatant was assembled and disassembled twice more to obtain a third cycle cold supernatant of active tubulin subunits, which was used immediately.

Quantitation of tubule polymerization was done according to the sedimentation assay described by Johnson and Borisy (24). Tubulin stocks were diluted into assembly buffer such that the final volume was 125 μ l. Samples were incubated in 5 \times 75-mm glass tubes in a temperature-controlled water bath at either 22° or 37°C for 1 h. Samples (25 μ l) were removed for a total protein determination. Microtubule assembly was qualitatively monitored by the development of birefringence observed with a hand-held polaroscope. Tubules were pelleted by centrifugation at 27,000 g for 1 h at the polymerization temperature. The supernatants were carefully removed for protein determinations. The pellets were resuspended in 100 μ l of assembly buffer, depolymerized on ice for 30 min, and centrifuged at 27,000 g to obtain a supernatant containing active polymer. Protein determinations were done on the active polymer fraction. All data were plotted by linear regression analysis. Protein concentrations were determined by the method of Lowry (37) as modified by Bensadoun and Weinstein (5).

Light Microscopy: The isolation and purification of *Spisula* meiotic spindles was monitored using a Zeiss microscope equipped with polarization optics, a xenon light source, and heat absorbing filters. The assembly of surf clam tubulin was monitored by dark field light microscopy using a Zeiss microscope equipped with a Zeiss oil immersion ultracondensor (NA 1.2/1.4). Preparations were illuminated with a 1,000-W xenon lamp (Hanovia XBO 1000; Oriel Corporation of America, Stamford, CT) in an Oriel lamp housing with a 48 mm fl.0 silica condensing lens and an Oriel 6242 power supply (Oriel Corporation of America).

Electrophoresis: Proteins were resolved on one-dimensional polyacrylamide gels according to the method of Laemmli (32). Gels were stained with Coomassie Blue according to the method of Fairbanks et al. (17). Isoelectric focusing and two dimensional electrophoresis were carried out using O'Farrell's

¹ Abbreviations used in this paper: Ccr, critical concentration; MAPs, microtubule-associated proteins; PC, phosphocellulose column.

procedures (43); the first dimension isoelectric focusing gels were done on 0.75-mm slab gels. Individual lanes were cut out and run in the second dimension on 1.5-mm SDS polyacrylamide gels with a 4–16% gradient in acrylamide (32). Proteins were visualized on the isoelectric focusing gels by staining with Coomassie Blue (17) after the gel had been fixed 1 h in 12.5% trichloroacetic acid and 12 h in 45% isopropanol, 10% acetic acid. Proteins were visualized in the second dimension by fixation of the proteins with 45% methanol, 12.5% acetic acid, followed by silver staining according to the method of Merrill et al. (38).

One-Dimensional Peptide Mapping: One-dimensional limited proteolysis of heterogeneous protein samples was done on SDS polyacrylamide gels according to the method of Bordier and Crettol-Jarvinen (7). Unfertilized oocyte and meiotic spindle tubulin, after three cycles of assembly (5 μ g each) were electrophoresed on 0.75-mm-thick 7.5% polyacrylamide gels (32). Individual lanes were cut out and equilibrated in Laemmli sample buffer without mercaptoethanol. For the limited proteolysis, the lanes were placed on their side and layered on a 1.5-mm SDS polyacrylamide gel with a 3% stacking gel and a 15% acrylamide separating gel. The gel was overlaid with sample buffer containing 0.4 μ g *Staphylococcus aureus* V8 protease, and electrophoresed at 100 V until the dye front was 1 cm into the stacking gel. Electrophoresis was stopped for 2 h while proteolysis took place in the stacking gel. The resulting peptides were electrophoresed at 50 V for ~15–18 h or until the dye front had run to the bottom of the separating gel. Proteins were fixed in the gel with 45% methanol and 12.5% acetic acid and stained using the silver technique modified by Merrill et al. (38).

Electron Microscopy: Microtubule pellets were fixed with assembly buffer containing 0.5% glutaraldehyde and 0.5, 2.0, or 6.0% tannic acid (concentration where noted) (4). Assembly buffer was neutralized with NaOH, not KOH, to prevent precipitation of tannic acid. The material was fixed at room temperature for 6–9 h, postfixed for 30 min with 0.5% osmium tetroxide in 0.1 M sodium phosphate buffer, pH 6.8, stained en bloc with 1% aqueous uranyl acetate for 1–3 h, dehydrated in ethanol and propylene oxide, and embedded in Epon-Araldite. Thin sections were stained with 1% methanolic uranyl acetate (50% methanol) and lead citrate (64). Microtubules were also negatively stained with 1% uranyl acetate on carbon-coated formvar grids. Samples were examined with a Hitachi HU-11E1 or a Philips EM 300.

RESULTS

Purification of Assembly-competent Tubulin from Oocytes and Meiotic Spindles

Assembly-competent tubulin was obtained by the methods described above from the total pool of cytoplasmic tubulin at two developmental stages; unfertilized oocytes and parthenogenetically activated oocytes at first meiotic metaphase. Fig. 1 illustrates the purification of cytoplasmic tubulin from unfertilized oocytes (A) and activated oocytes at meiotic metaphase (B). Both preparations were very similar as analyzed by SDS polyacrylamide gel electrophoresis. Most of the non-tubulin proteins were washed from the column with 0.35 M NaCl (lane C, Fig. 1) whereas the oocyte tubulin was eluted from the DEAE-Sephadex column with 0.65 M NaCl (lane D, Fig. 1). The tubulin-containing fractions were concentrated by ammonium sulfate precipitation, desalted into microtubule assembly buffer, and further purified by temperature-dependent cycles of assembly and disassembly (lanes 1x, 3x, Fig. 1). The yield of assembly-competent cytoplasmic tubulin from 4 ml of packed oocytes was 2–4 mg. Much lower yields were obtained from larger preparations. For example, only 6–8 mg of tubulin was obtained from a DEAE-preparation starting with 20–30 ml of packed oocytes.

Meiotic spindle tubulin was isolated from parthenogenetically activated *Spisula* oocytes by the methods of Keller and Rebhun (25). In this procedure, meiotic spindle tubulin was obtained from isolated hexylene glycol-augmented spindles. The spindle microtubules were depolymerized by cold and the tubulin was recovered in the cold supernatant after centrifugation of the spindle remnants. The soluble tubulin was further purified by cycles of temperature-dependent assembly

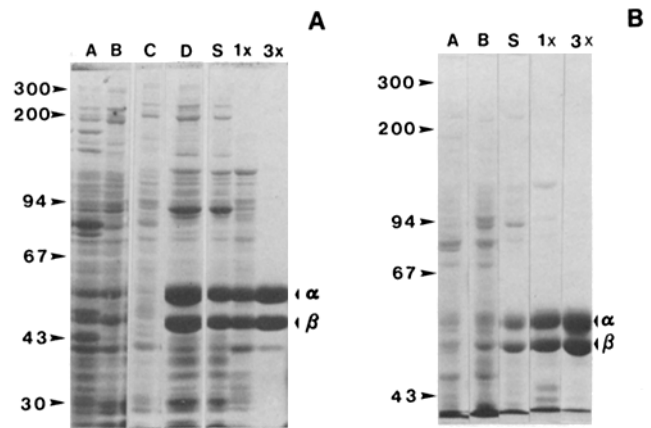


FIGURE 1 Purification of cytoplasmic tubulin from unfertilized and parthenogenetically activated surf clam oocytes by DEAE column chromatography and cycles of assembly and disassembly. (A) Unfertilized oocytes; 7.5% SDS PAGE stained with Coomassie Blue. (B) Parthenogenetically activated oocytes; 5% SDS PAGE stained with Coomassie Blue. Lane A, total protein in 100,000-g supernatant; lane B, protein loaded on DEAE-Sephadex; lane C, protein washed from column with 0.35 M NaCl; lane D, concentrated tubulin containing fractions eluted from DEAE with 0.65 M NaCl; lane S, warm supernatant after first cycle of polymerization; lane 1x, first cycle polymer at 37°C; lane 3x, third cycle polymer at 37°C.

and disassembly (lanes 1x, 2x, 3x, Fig. 2). The yield of assembly-competent meiotic spindle tubulin from 4 ml of packed oocytes was 0.25–0.50 mg.

In Vitro Assembly of Macrotubules from Surf Clam Tubulin

Polymerization of the three different tubulin samples was monitored by dark field light microscopy or by the development of birefringence observed with a polarization light microscope. After warming a preparation to 22° or 37°C for 15–30 min, very bright refractile bundles of polymerized material were observed by dark field light microscopy (Fig. 3A). Electron microscopy of thin sections of this material, assembled at either temperature, revealed that the polymer formed 48–51-nm (outer diameter) macrotubules (Fig. 3C). In preparations examined with dark field light microscopy and in thin sections examined by electron microscopy, the macrotubules were found very close together in bundles, and appeared almost crystalline in some cross-sections (Fig. 3, A and C). Macrotubule walls appeared fused and it was difficult to tell where individual macrotubules began. No protofilaments were seen in cross-sections of fixed and sectioned material.

In negatively stained preparations (Fig. 3B), the protofilaments of the macrotubules were coiled at a ~36° angle to the long axis of the macrotubule. The number of protofilament pairs that assembled to form a macrotubule was calculated as a function of the macrotubule diameter, the protofilament angle to the long axis, and the protofilament pair width (65). Using a 50-nm average macrotubule diameter, determined from cross-sections of fixed and embedded surf clam macrotubules, and a protofilament pair width of 10 nm (16), the calculated number of protofilament pairs found in in vitro assembled macrotubules was 12.7. The experimentally determined data agreed with the calculated values such that in open sheet regions (Fig. 3B), 26 or 27 protofilaments were

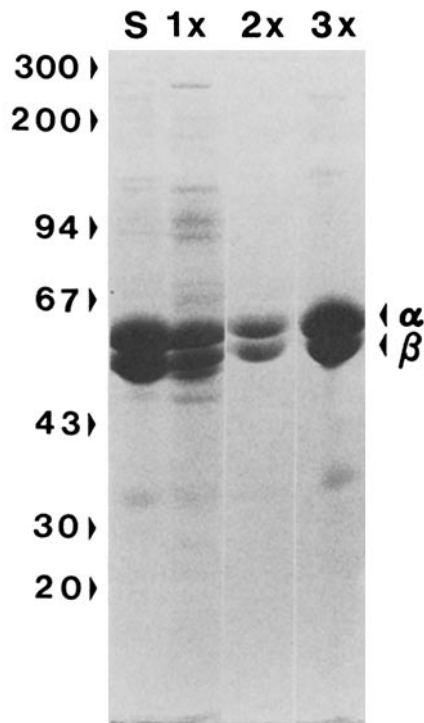


FIGURE 2 Purification of surf clam meiotic spindle tubulin by cycles of assembly and disassembly at 37°C (4–16% SDS PAGE stained with Coomassie Blue). Lane S, warm supernatant after the first cycle of assembly; lane 1x, first-cycle polymer; lane 2x, second-cycle polymer; lane 3x, third-cycle polymer.

observed. It is not known whether the open ribbon structures observed in negatively stained preparations were intermediate structures in the formation of microtubules or whether they resulted from the process of negative staining.

In vitro assembled microtubules were cold labile, such that the birefringent highly refractile bundles disappeared within 60 s when 10 μ l of the preparation on a microscope slide was cooled to 4°C. The microtubule proteins were very stable and could be stored in a refrigerator for up to 48 h without losing their ability to polymerize. Preparations of microtubules within sealed microscope slides could be polymerized at room temperature and depolymerized at 4°C for up to 12 times over a 2-d period, even in the absence of additional GTP.

The major proteins found in the microtubule preparations during the first cycle of assembly were α and β tubulins (lane 1x, Figs. 1 and 2). Several unidentified proteins with molecular weights ranging from 30,000 to 250,000 were also present in microtubule preparations as assayed by SDS polyacrylamide gel electrophoresis (lanes 1x, Figs. 1 and 2).

While microtubules were the predominant polymer formed during the first cycle of polymerization, 25-nm microtubules were also found simultaneously with the microtubules at steady state. Steady state plateau values were obtained by monitoring spectrophotometrically the change in solution turbidity at 350 nm (18). We found no reliable way to quantitate the number of microtubules or microtubules present in first cycle preparations, although from negatively stained preparations examined by electron microscopy 1 h after reaching steady state plateau values, we estimated that 75–95% (by number) of the tubules formed from unfertilized oocyte tubulin were 50-nm microtubules. The percentage of microtubules assembled from tubulin isolated from parthen-

ogenetically activated oocytes or meiotic spindles was much less than the percentage observed with unfertilized oocyte tubulin. Only 25–50% of the total polymer in these preparations were microtubules, as estimated by observation of negatively stained preparations.

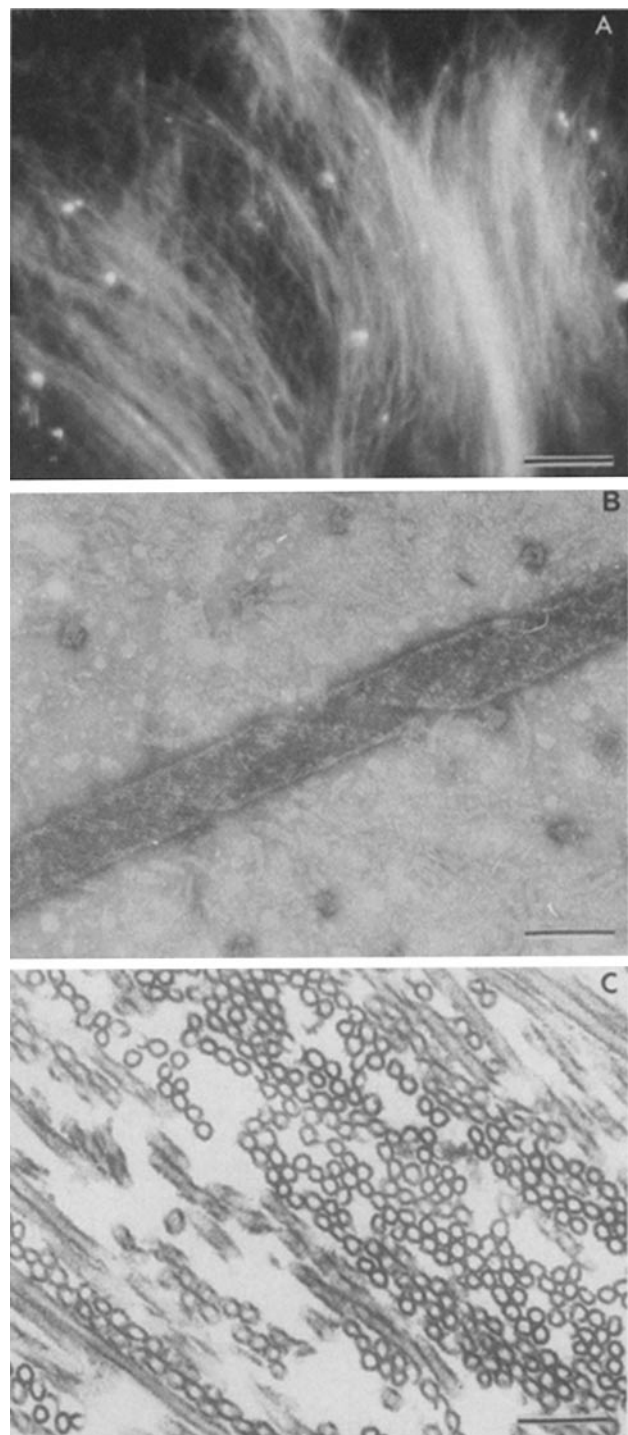


FIGURE 3 First cycle of surf clam tubulin assembly at 37°C. (A) Darkfield light micrograph of polymerized *Spisula* oocyte tubulin; (B) negatively stained microtubules assembled during the first cycle of assembly of *Spisula* meiotic spindle tubulin; (C) thin section of microtubules assembled during the first cycle of assembly of *Spisula* oocyte tubulin and fixed in the presence of 6% tannic acid. Bars, 12.5 μ m (A); 0.11 μ m (B); 0.25 μ m (C).

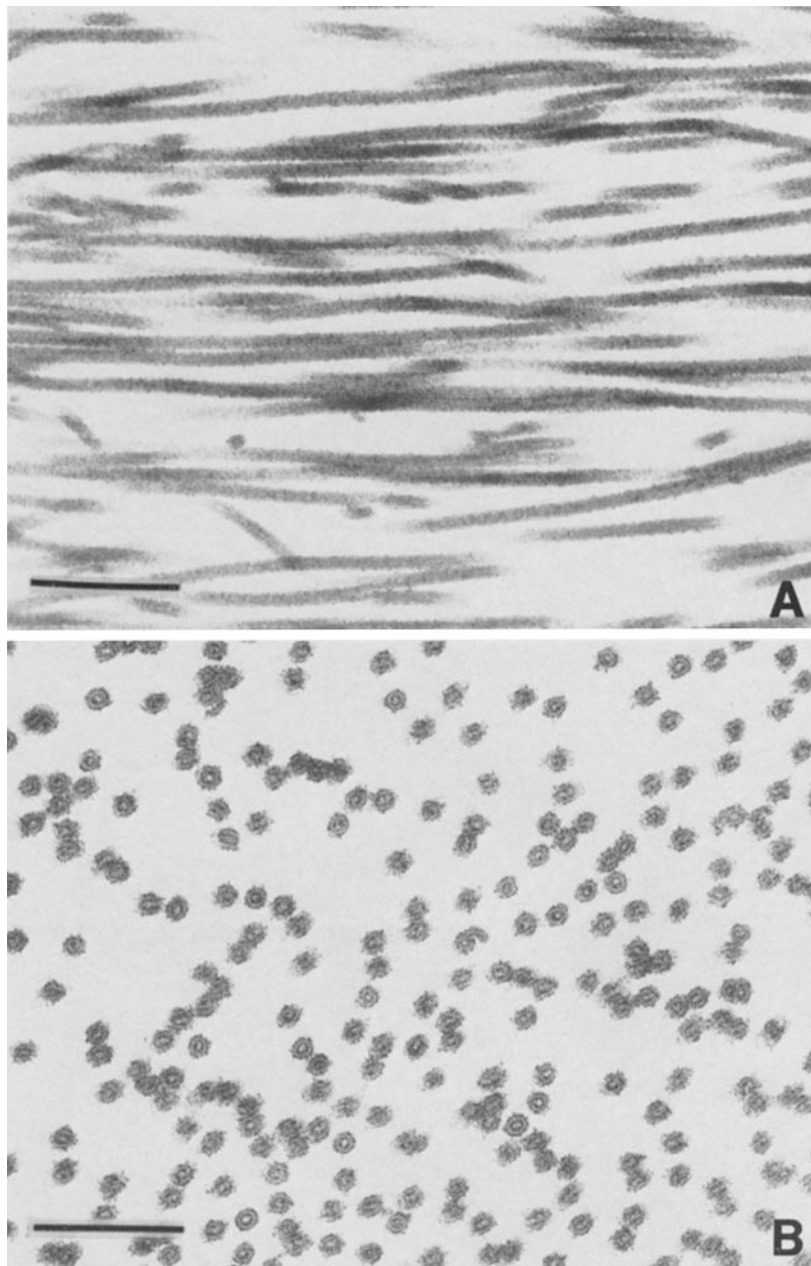


FIGURE 4 Thin section of microtubules assembled during third cycle of polymerization of DEAE chromatographed unfertilized oocyte tubulin fixed in the presence of 6% tannic acid. (A) longitudinal section; (B) cross-section. Bars, 0.29 and 0.22 μm , respectively.

In Vitro Assembly of Microtubules from Surf Clam Tubulin

Surf clam tubulin was further purified by cycles of temperature-dependent assembly and disassembly. By the third cycle of assembly, microtubules comprised >95% of the tubules formed (Fig. 4), and, usually, microtubules were the exclusive polymer formed during the third and subsequent cycles of assembly. Nevertheless, there was some variation among preparations such that macrofibrils were occasionally observed in third cycle preparations, as seen in Fig. 5, where macrofibrils were present (~10% by number) in a third cycle preparation composed predominantly of microtubules. Macrofibrils were easy to distinguish from the microtubules by their large diameter and the filamentous halo of material visible on

the surface of the macrofibril in both longitudinal and cross-sections (Fig. 5, A-C). The microtubules in this fixed and embedded material were smooth walled with no filamentous projections.

The protein composition of the polymer formed during the third cycle of assembly was nearly homogeneous. Third-cycle microtubule preparations from unfertilized and parthenogenetically activated oocytes were >95% α and β tubulins (lane 3x, Fig. 1) with trace amounts of a low molecular weight protein with an apparent molecular weight of 39,000 on these gels. Third cycle microtubules assembled from isolated meiotic spindle tubulin were composed of predominantly tubulin with trace amounts of three proteins with apparent molecular weights of 250,000, 150,000, and 33,000 (lane 3x, Fig. 2).

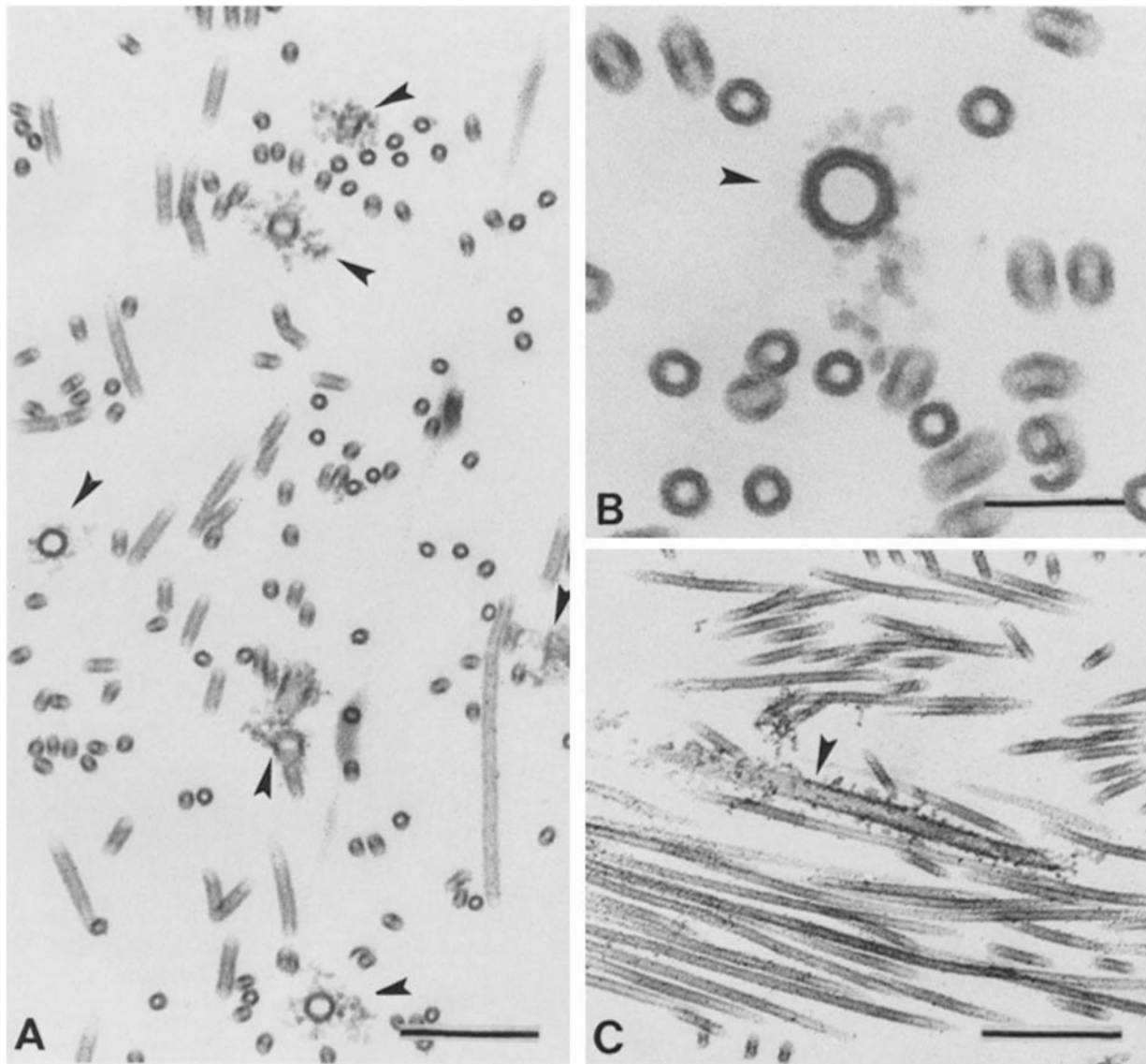


FIGURE 5 Thin section of microtubules and macro tubules assembled during the third cycle of assembly of *Spisula* oocyte tubulin fixed in the presence of 0.5% tannic acid. Arrows point to macro tubules. Bars, 0.32 μm (A); 0.11 μm (B); 0.38 μm (C).

Phosphocellulose Chromatography of Surf Clam Oocyte Tubulin

Cation exchange resins such as phosphocellulose will bind microtubule-associated proteins from brain microtubule preparations leaving pure tubulin in the flow-through fractions (53, 67). For these experiments, unfertilized *Spisula* oocyte tubulin was isolated by DEAE-column chromatography. One half of the sample was warmed at 37°C to assemble macro tubules (Fig. 6, A and B) and the other half was chromatographed on a phosphocellulose column (PC). Tubulin-containing fractions, obtained from PC chromatography (Fig. 6A), assembled exclusively into microtubules (Fig. 6C). No macro tubules were observed in negatively stained PC preparations or in pellets fixed and thin sectioned for electron microscopy. The tubulin sample, that was not chromatographed on PC, assembled into macro tubules (Fig. 6B). PC chromatography removed several nontubulin proteins present in the DEAE sample, ranging in molecular weight from

60,000 to 110,000, leaving only α and β tubulin in the flow-through fractions (Fig. 6A).

Sedimentation Assay for Microtubule Preparations

It is known from studies of brain tubulin assembly that tubulin dimers are in equilibrium with the microtubule polymer at steady state (18, 24). The equilibrium concentration of tubulin dimers is known as the critical concentration (Ccr). Below the Ccr no assembly takes place, and above this protein concentration, microtubules are readily formed (18, 24). The true critical protein concentration necessary for microtubule polymerization can be experimentally determined using the sedimentation assay described by Johnson and Borisy (24) for the polymerization of brain microtubule protein in vitro. Using cycle purified brain microtubule protein (tubulin plus MAPs), we obtained a Ccr of 0.15 mg/ml at 37°C (data not shown), a value that agrees with the published values of Johnson and Borisy (24) using this technique, and with other

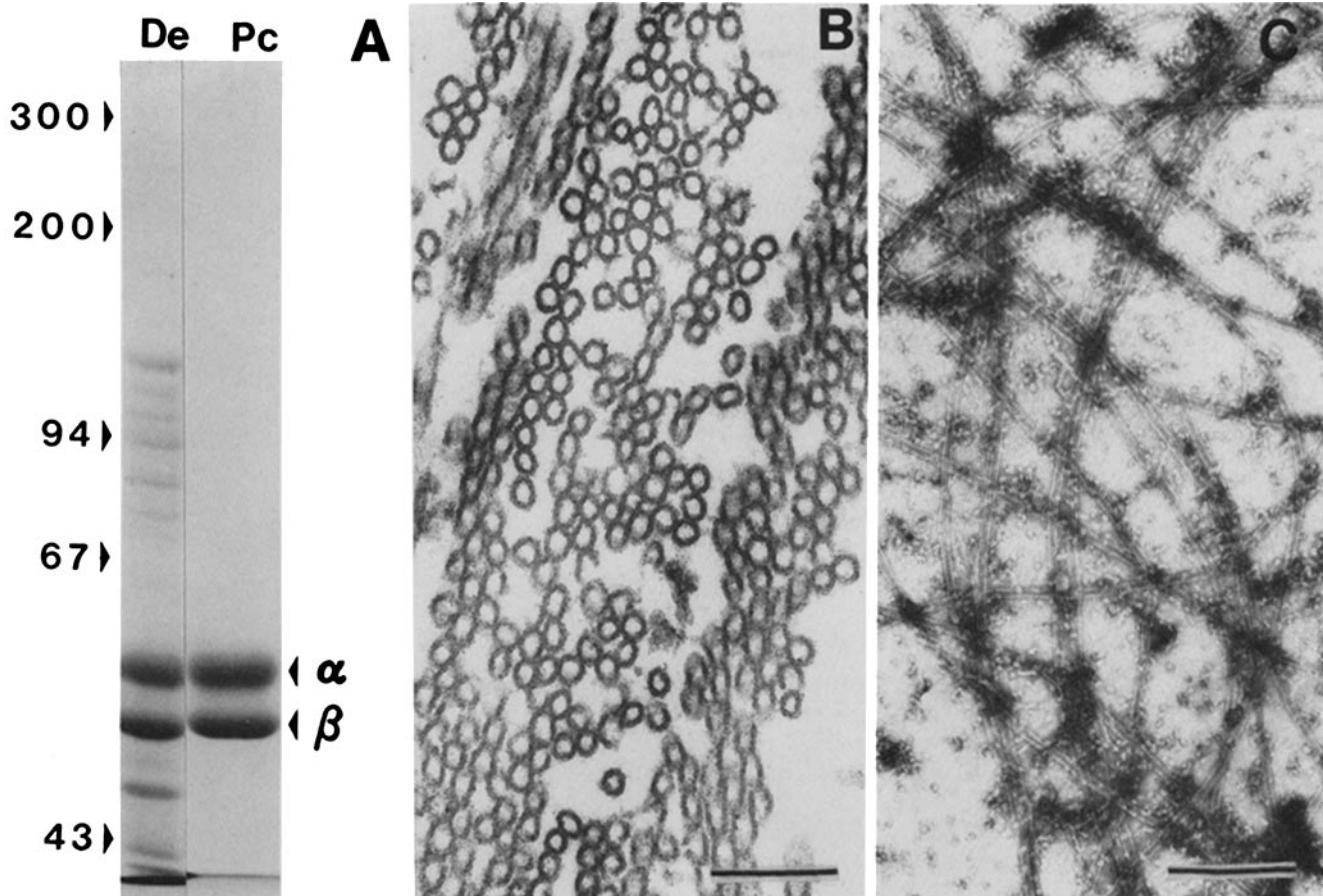


FIGURE 6 Assembly and purification of phosphocellulose-purified surf clam tubulin. (A) 5% SDS PAGE stained with Coomassie Blue. Lane *De*, proteins associated with first cycle macro-tubules assembled from DEAE-purified oocyte tubulin; lane *Pc*, phosphocellulose-purified *Spisula* oocyte tubulin that assembled into microtubules. (B) Thin section of macro-tubules assembled from DEAE-purified surf clam tubulin and fixed in the presence of 6% tannic acid. (C) Negatively stained surf clam microtubules assembled from phosphocellulose-purified oocyte tubulin. Bars, 0.25 μ m (B); 0.43 μ m (C).

turbidometric and viscometric determinations of the apparent Ccr (18, 44). The technique was applied to the assembly of *Spisula* oocyte tubulin as described below.

Assembly of Surf Clam Cytoplasmic Tubulin at 37°C

Surf clam tubulin, at 1 mg/ml, self-assembled rapidly at 37°C and reached steady state turbidity values in 7–12 min. Plateau values were determined spectrophotometrically by the measured increase in turbidity at 350 nm (18, data not shown). The polymer formed was cold labile and the birefringence observed with a hand held polaroscope disappeared within 5 min of placing the cuvette on ice.

The assembly of third cycle unfertilized oocyte tubulin and activated oocyte tubulin was compared by the sedimentation assay described above. Surf clam tubulin from both activated and unfertilized oocytes self-assembled at 37°C in a concentration-dependent manner at a critical protein concentration of 0.09 mg/ml (range of 0.04–0.15 mg/ml, four determinations) (Table 1). Although the Ccr of unfertilized and activated oocyte tubulin were identical, the slopes of the supernatant curves were quite different, and thus, either unfertilized oocyte tubulin was denatured preferentially at 37°C or a greater percentage of activated oocyte tubulin was able to polymerize.

Assembly of Surf Clam Oocyte Tubulin and Spindle Tubulin at Physiological Temperatures

Surf clam embryos will not grow at 37°C, therefore, further *in vitro* assembly studies were done at 22°C, a temperature physiological for these animals. The first cycle of assembly of DEAE-purified unfertilized oocyte tubulin was compared with the third cycle of assembly by the sedimentation assay. The critical concentration for the first cycle of tubulin polymerization at 22°C was 0.48 mg/ml (Table I). The slope of the supernatant curve was 0.12, indicating that 88% of the total protein was active in the polymerization process. Third cycle unfertilized oocyte tubulin self-assembled at a Ccr of 0.36 mg/ml at 22°C (Fig. 7A). The Ccr for the first cycle of assembly was slightly higher than the Ccr for the third cycle of assembly. Third cycle activated-oocyte tubulin assembled at a Ccr of 0.42 mg/ml (Fig. 7B) and third cycle meiotic spindle tubulin assembled in a concentration-dependent manner at a Ccr of 0.33 mg/ml (Fig. 7C). The Ccr necessary for the assembly of unfertilized or activated oocyte cytoplasmic tubulin or meiotic spindle tubulin was nearly identical, differing by no more than 0.09 mg/ml at 22°C. In addition, the slopes of the supernatant curves were very similar, ranging from 0.17–0.25. This indicated that in all three tubulin preparations, 75–83% of the total microtubule protein was active

TABLE I
Critical Concentrations for Assembly of *Spisula* Oocyte and
Spindle Tubulins

Sample	Tubulin alone		Tubulin + MAPs	
	3X @ 37°C	1X @ 22°C	3X @ 22°C	3X @ 22°C
Oocyte cytoplasmic tubulin	.09 (.59)	.48 (.12)	.36 (.17)	.21 (.03)*
Activated oocyte cytoplasmic tubulin	.09 (.35)	—	.42 (.23)	.21 (.14)*
Meiotic spindle tubulin	—	—	.33 (.25)	—

The critical concentrations for polymerization of *Spisula* tubulin was determined by the sedimentation assay described in Materials and Methods. The slopes of the supernatant curves, indicative of the percentage of active subunits, are in parentheses. 1X, first cycle of polymerization; 3X, third cycle of polymerization.

* Tubulin was co-assembled with heat-stable high molecular weight MAP-2 isolated from bovine brain microtubule protein (29).

* Tubulin was co-assembled with bovine brain MAPs purified by phosphocellulose chromatography (53, 67).

in the polymerization process. Meiotic spindle tubulin had the lowest activity, 75%, which may reflect the presence of the 250,000, 150,000, and 33,000-mol wt nontubulin proteins found in these preparations. In addition, the slopes of the supernatant curves at physiological temperatures were much lower than at 37°C (compare slopes at 37° and 22°C, Table I). These data indicate that egg tubulin possessed a higher percentage of polymerizable subunits at physiological temperatures.

Comparison of Oocyte Cytoplasmic Tubulin and Meiotic Spindle Tubulin by Isoelectric Focusing and One-Dimensional Peptide Mapping

To look at the purity of the preparations and to compare the potentially different pools of tubulin, oocyte tubulin and meiotic spindle tubulin were analyzed by isoelectric focusing and two-dimensional electrophoresis. Isoelectric focusing (43) was done on vertical slab gels to directly compare protein samples on adjacent lanes. Third-cycle meiotic spindle tubulin and unfertilized oocyte cytoplasmic tubulin co-migrated on isoelectric focusing gels with a pI of 5.8 for α tubulin and 5.6 for β tubulin (Fig. 8). Both α and β tubulin subunits from the oocyte cytoplasm and meiotic spindles were identical in their isoelectric points. The purity of the preparations and the identity of the isoelectric species were confirmed by two-dimensional gel electrophoresis. No other proteins were observed to focus with the same molecular weight as α and β tubulin in either preparation (Fig. 8).

Third-cycle oocyte tubulin and meiotic spindle tubulin were compared by one-dimensional peptide mapping. Proteins were digested with the enzyme *S. aureus* V8 protease according to the method of Bordier and Crettol-Jarvinin (7). Peptides were visualized on the one-dimensional maps by the sensitive silver staining technique (38), allowing the visualization of proteolytic digests with as little as 2–3 μ g of starting protein. The β -subunits were cleaved into seven peptides that were resolved on the 15% acrylamide gels whereas the α -subunits were cleaved into two predominant peptides (Fig. 9). The peptide maps of α and β tubulins from the oocyte cytoplasm

and isolated meiotic spindles were qualitatively very similar, if not identical.

Assembly of Surf Clam Oocyte Tubulin in the Presence of Bovine Brain MAPs

For these experiments, unfertilized surf clam tubulin was purified by DEAE chromatography and three cycles of temperature-dependent assembly and disassembly. Bovine brain MAPs were obtained by phosphocellulose chromatography of twice-cycled bovine brain microtubule proteins (53, 67). Quantitation of microtubule assembly was done at 22°C by the sedimentation assay. Surf clam oocyte tubulin (0.3–0.6 mg/ml) was assembled in the presence of 0.5 mg/ml phosphocellulose-chromatographed brain MAPs. Extrapolation of the supernatant curves to infinite dilution indicated a Ccr of 0.21 mg/ml (Fig. 10). The experiments were repeated with the heat stable MAP-2 alone, purified by the procedures of Kim et al. (29). Again, surf clam oocyte tubulin assembled at a Ccr of 0.21 mg/ml (Table I) (mass ratio of MAP-2 to tubulin = 2:1). Surf clam tubulin, in the presence of PC brain MAPs or heat stable MAP-2 assembled at a twofold lower protein concentration than surf clam tubulin alone.

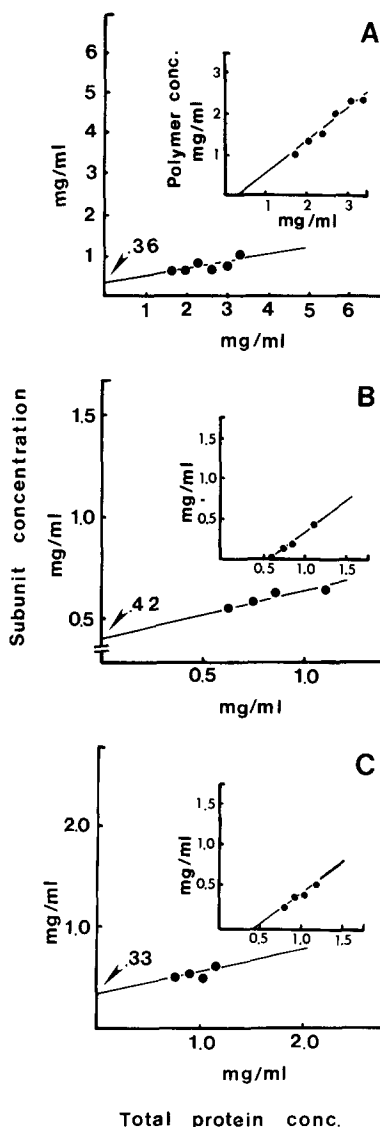


FIGURE 7 Quantitation of third cycle microtubule assembly at 22°C by sedimentation assay. All data was plotted by linear regression analysis in the form subunit concentration (ordinate) vs. total protein concentration (abscissa); insets, polymer formation (ordinate) vs. total protein concentration (abscissa). (A) Assembly of unfertilized oocyte tubulin. Extrapolation of subunit concentration to infinite dilution indicates a critical concentration of 0.36 mg/ml. The slope of the curve is 0.17. (B) Assembly of activated oocyte tubulin. Critical concentration is 0.42 mg/ml and the slope of the curve is 0.23. (C) Assembly of meiotic spindle tubulin. Critical concentration is 0.33 mg/ml and the slope of the curve is 0.25.

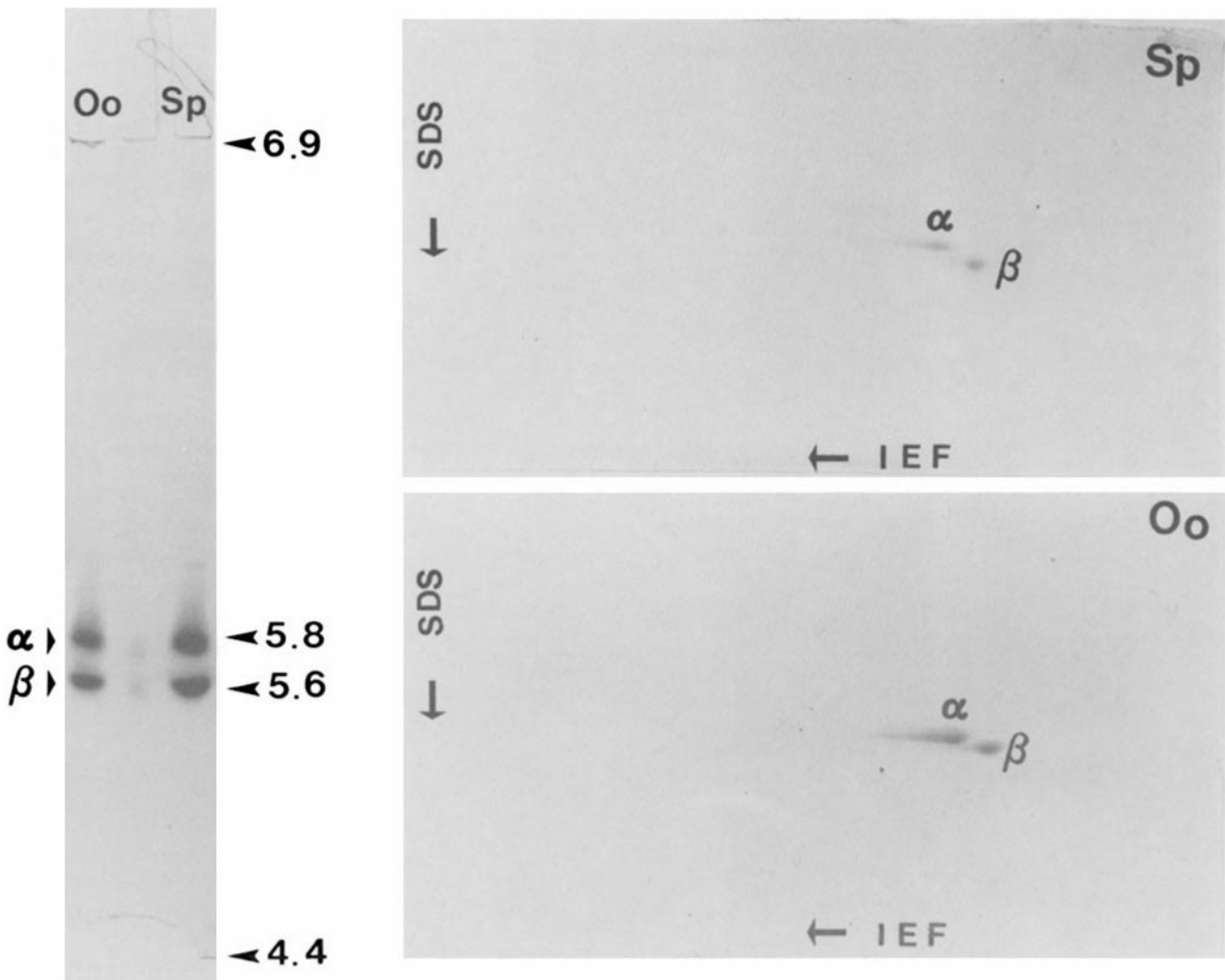


FIGURE 8 Isoelectric focusing and two dimensional gel electrophoresis of surf clam tubulin. Isoelectric focusing gels were stained with Coomassie Blue. Two dimensional gels contained a 4–16% acrylamide gradient and were silver stained (see Materials and Methods). Lane and gel Oo, third cycle unfertilized oocyte tubulin; lane and gel Sp, third cycle meiotic spindle tubulin.

The polymer formed by the co-assembly of brain MAPs and surf clam tubulin was pelleted and processed for electron microscopy. At steady state (determined spectrophotometrically), and up to 60 min after reaching steady state, brain MAPs induced almost exclusively the formation of coiled ribbon polymers (Fig. 11, *B* and *C*). Intact microtubules were rarely observed in these preparations. Coiled ribbon polymers were formed by the co-assembly of brain MAPs with DEAE-purified tubulin from either unfertilized oocytes or parthenogenetically activated oocytes at 22° or 37°C. Meiotic spindle tubulin was not tested for assembly in the presence of brain MAPs.

The coiled ribbons were composed primarily of tubulins and the high molecular weight MAP-2 as shown in Fig. 12 (lane *P*). The coiled ribbons were cold labile and disappeared within 5–10 min at 4°C. However, microtubules or coiled ribbons would not assemble a second time from the cold-labile soluble proteins. Centrifugation of the cold depolymerized coiled spirals removed a sedimentable aggregate of surf clam tubulin and brain MAPs apparently necessary for further cycles of assembly.

DISCUSSION

Assembly of Macrotubules from Surf Clam Tubulin

Cytoplasmic microtubules *in vivo* from various sources are composed of 13 protofilaments (12, 47, 61), although as few as 12 protofilaments have been observed in crayfish axons (12) and as many as 15 in cockroach epidermal cells (41). In most cases, *in vitro* assembled microtubules lose the ability to maintain a 13-protofilament structure during repeated cycles of assembly and disassembly (47, 51). With the exception of yeast microtubules (28), *in vitro* assembled microtubules are composed of 14 and 15 protofilaments (6, 29, 34, 47, 51). Variations in the number of protofilaments *in vivo* and *in vitro* may be a reflection of the observed differences in microtubule biochemistry and function (14). Changes in the number of protofilaments may alter the association of various proteins with the microtubule surface, thereby altering the axial spacing of the various links and cross-bridges observed between some microtubules (2).

Surf clam tubulin assembled into 26–27 protofilament macrotubules during the first cycle of assembly following DEAE

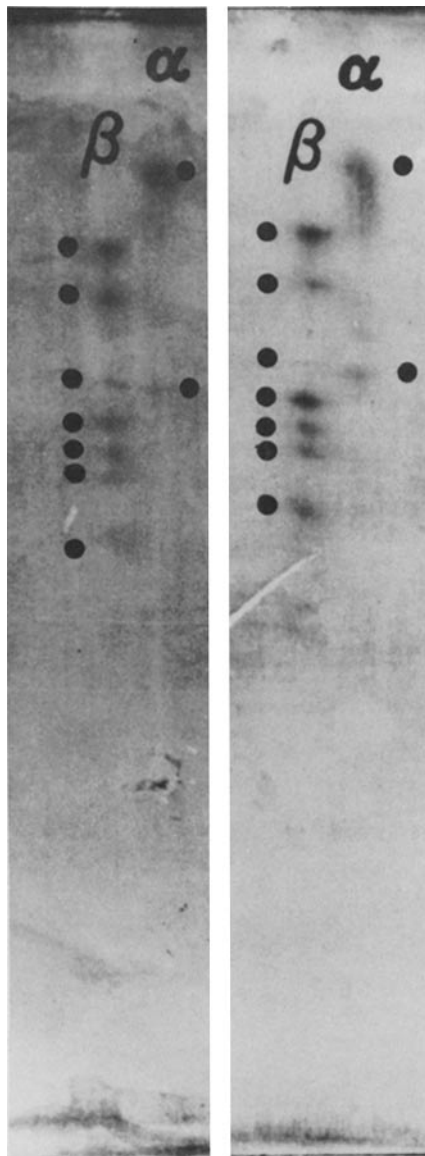


FIGURE 9 *S. aureus* protease cleavage patterns for α and β tubulins from *Spisula* oocytes (left) and meiotic spindles (right). The subunits were digested by *S. aureus* V8 protease in a 3% stacking gel and peptides were resolved by electrophoresis on a 15% polyacrylamide gel. Both oocytes and spindle tubulins were mapped on the same gel. The photograph has been cropped to place the samples closer together. Dots indicate the position of the major cleavage products.

chromatography and 14–15 protofilament microtubules during the third and subsequent cycles of *in vitro* assembly. Although there are no known reports of a naturally occurring tubulin polymer resembling a macro-tubule, there are several reports describing the presence of short macro-tubules within cells, induced by experimental manipulation. Macro-tubule formation has been reported in rat renal cells in the presence of vinblastine or colchicine (62), in axopodia of the heliozoan *Echinospaerium*, induced by low temperature or colchicine (58, 60, 66), plant cells in the presence of digitonin (20), crayfish nerve cord by the addition of hyaluronidase (11) or halothane (21, 22) and in the unicellular flagellate *Ochromonas* induced by high pressure and isopropyl *N*-phenyl carbamate (IPC) (8). In most cases, microtubules disappear

with the concomitant reappearance of short macro-tubules embedded in a granular and filamentous matrix. These investigators have suggested that macro-tubules may be a storage form of tubulin or a microtubule assembly intermediate. Microtubules reappear and macro-tubules disappear when the disrupting agent is washed out. There are no reports of macro-tubule assembly under buffer conditions that would normally support microtubule assembly.

A comparison of the protein composition and assembly properties of macro-tubules and microtubules from surf clam tubulin indicated that a nontubulin component might be responsible for macro-tubule formation. *In vitro* assembly studies indicated that macro-tubules and microtubules required approximately the same tubulin concentration to assemble since the critical concentrations for the first and third cycles of surf clam tubulin assembly were very similar. Further, macro-tubules and microtubules were present simultaneously an hour after reaching steady state (steady state plateau values were determined spectrophotometrically). If the critical concentrations for the assembly of each polymer were different, the polymer with the greater critical concentration would depolymerize at the expense of the other polymer because polymerization would always reduce the equilibrium subunit concentration to the lowest critical concentration. It is possible, however, that equilibrium was not attained and one polymer was slowly depolymerizing, such that the

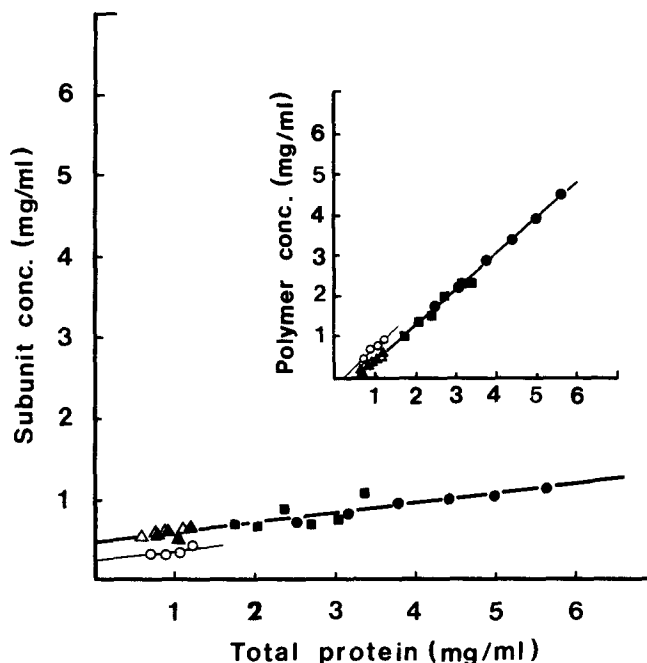


FIGURE 10 Quantitation of surf clam tubulin assembly in the presence of phosphocellulose purified brain MAPs at 22°C. All data graphed by linear regression analysis as subunit concentration vs. total protein concentration (inset). Polymer formed vs. total protein concentration. Surf clam activated oocyte tubulin assembled in the presence of 0.5 mg/ml brain MAPs. Extrapolation of the supernatant curve (O) to infinite dilution indicates a critical concentration of 0.21 mg/ml. Slope of the curve is 0.14. Pooled data for the assembly of surf clam tubulin at 22°C in the absence of MAPs. Extrapolation of the supernatant curve to infinite dilution indicates a Ccr of 0.46 mg/ml. The slope of the curve is 0.14 and the linear regression coefficient is 0.99. ●, first cycle and ■, third cycle of unfertilized oocyte tubulin assembly; ▲, third-cycle meiotic spindle tubulin assembly and Δ, third-cycle activated oocyte tubulin assembly.

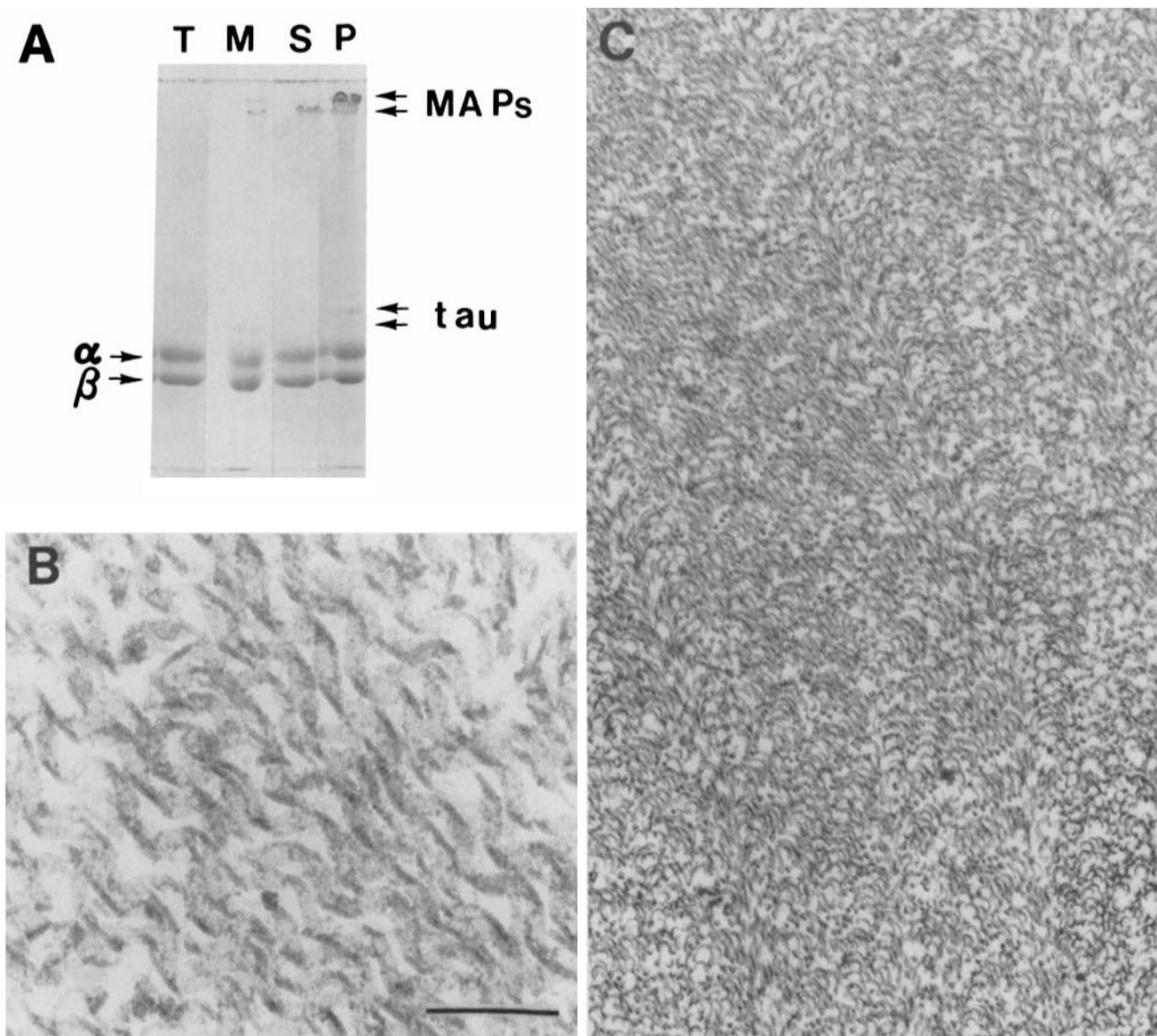


FIGURE 11 Assembly of surf clam tubulin in the presence of bovine brain MAPs. (A) 5% SDS polyacrylamide gel stained with Coomassie Blue. Lane *T*, surf clam oocyte tubulin; lane *M*, bovine brain MAPs and surf clam tubulin prior to warming; lane *S*, warm supernatant after the first cycle of polymerization at 22°C; lane *P*, proteins associated with coiled ribbon polymers assembled during the first cycle of assembly of surf clam tubulin and bovine brain MAPs. (B) Thin section of coiled ribbon polymers assembled from surf clam oocyte tubulin and bovine brain MAPs and fixed in the presence of 0.5% tannic acid. Bar, 0.20 μ m. (C) Low magnification of thin section of coiled ribbon polymer showing absence of any microtubule assembly.

conversion of a preparation entirely into either macro tubules or micro tubules would take longer than 1 h. Further assembly experiments with longer time courses are necessary to accurately determine whether the observed distributions of macro tubules and micro tubules are stable at observed steady state turbidity levels.

Surf clam macro tubule preparations contain in addition to tubulin, several proteins with molecular weights of 30,000–270,000. Removal of these nontubulin proteins by either further cycles of assembly and disassembly or by phosphocellulose chromatography resulted in a preparation that assembled micro tubules. However, cations such as zinc or calcium have also been shown to induce sheet or ribbon formation with tubulin. For example, zinc ions induced mammalian

brain tubulin to form sheets with up to 60 protofilaments, which in thin sections, are suggestive of a 200-nm macro tubule structure (19, 35). In addition, Langford (34) described the assembly of large coiled ribbon polymers from dogfish brain tubulin in the presence of calcium. Thus, our present data indicates that either a protein(s) or cation(s), such as calcium or zinc that might be removed by phosphocellulose (71) or dilution during repeated polymerization-depolymerization cycles, is responsible for macro tubule formation. Such factors that alter the assembly properties of the tubulin molecule *in vitro* are potentially important regulators of micro tubule biochemistry and function *in vivo*. Further work is in progress to isolate and characterize the factors involved in macro tubule formation from *Spisula* oocyte tubulin.

Surf Clam MAPs?

The combination of chromatographic procedures and cycles of assembly and disassembly used to purify surf clam oocyte tubulin removed potential MAPs from the tubulin preparation. The only nontubulin protein found associated with DEAE purified surf clam oocyte microtubules after three cycles was a 39,000-mol-wt protein. This protein was present in low amounts by the third cycle and did not cross-react with polyclonal antibodies prepared against bovine brain tubulin (unpublished data). It is possible that the 39,000-mol-wt protein is related to the assembly-promoting proteolytic fragment of bovine brain MAP-2 described by Vallee (63), although a similar 39,000-mol-wt protein found associated with third cycle microtubules assembled from tubulin purified from unfertilized sea urchin eggs (57) did not cross-react with antibodies prepared against MAP-2 (unpublished data). Similar low molecular weight proteins were described by Binder and Rosenbaum (6) in preparations of in vitro assembled sea urchin flagellar outer doublet tubulin. Proteins of similar molecular weight were not found in tubulin preparations from surf clam meiotic spindles (this report) or from *Strongylocentrotus purpuratus* or *Arbacia punctulata* meiotic spindle tubulin (26, 49), suggesting that the low molecular weight proteins may be important to the biochemistry of marine egg and flagellar tubulins and not spindle tubulins.

Surf clam meiotic spindle tubulin preparations contained trace amounts of three proteins with molecular weights of 250,000, 150,000, and 33,000 that co-assembled with the spindle tubulin. Murphy (40) has reported the presence of four proteins (doublet at 250,000, single bands at 140,000 and 30,000) that were solubilized when surf clam spindle microtubules were depolymerized and that co-assembled with spindle tubulin in vitro. Keller and Rebhun (25) have shown that as many as six nontubulin proteins, present in variable amounts, co-assembled with meiotic spindle tubulin. Removal of these proteins by phosphocellulose chromatography did not effect the final amount of microtubule polymerization (25). Therefore these proteins do not appear to be similar to the assembly-promoting MAPs found in brain microtubule preparations, although they may be associated specifically with spindle function in vivo.

Surf clam oocyte tubulin co-assembled with mammalian brain MAPs but the polymer formed differed from that resulting from the interaction of brain MAPs with sea urchin egg or mammalian brain tubulin. Brain MAPs, at saturation, lowered the Ccr for assembly of sea urchin egg (57) and mammalian brain tubulin (29) by 40-fold. Brain MAPs co-assembled with sea urchin (57) and brain tubulin (29) through successive cycles of assembly and disassembly as well as decorated the walls of the microtubules with filamentous projections (15, 29, 39, 53). On the other hand, brain MAPs increased the final extent of surf clam oocyte tubulin assembly by only twofold. Further brain MAPs did not co-assemble with surf clam tubulin for more than one cycle of assembly and coiled spiral polymers were formed rather than intact microtubules. Thus, while brain MAPs interact with surf clam tubulin, stimulating assembly and altering the polymer formed, the interaction appears to have been considerably altered when compared with that of sea urchin and brain tubulin. Nevertheless, the results suggest the presence of a potential regulatory site partially preserved over a wide evolutionary range.

Regulation of Microtubule Assembly In Vivo

The results reported in this manuscript indicate that factors other than tubulin are involved in regulating the amount of tubulin polymerized at various developmental stages of *Spisula* oocyte development. This includes both positive and negative control of microtubule polymerization. For example, unfertilized *Spisula* oocytes contain a large pool of tubulin that largely remains unassembled until microtubules of the first meiotic apparatus are assembled after fertilization. The ability of unfertilized *Spisula* oocyte tubulin to assemble at 0.36 mg/ml at 22°C in vitro suggests that nontubulin factors may prevent microtubule assembly in vivo. This assumes that the assembly experiments described above were done with a significant fraction of the total pool of tubulin and that the isolation procedures have not modified or activated the tubulin present. Based upon Burnside's estimates of the total pool of tubulin in *Spisula* oocytes ($2-2.4 \times 10 \mu\text{g/egg}$) (10), a minimum estimate of the tubulin available, under our conditions for harvesting oocytes would be 9.6 mg tubulin from 4 ml of packed oocytes. Routinely, the DEAE procedure yielded 2-4 mg of assembly-competent tubulin from 4 ml of eggs. This represents 21-42% of the total pool of soluble tubulin. Even if the 2-4 mg of tubulin isolated represented all the assembly-competent tubulin in the egg (remaining 6-8 mg of tubulin unable to assemble), the pool of assembly-competent tubulin available would be 0.5-1.0 mg/ml of eggs, a value still greater than the critical concentration necessary for polymerization in vitro.

We suggest that oocyte tubulin is maintained in the unassembled state by a combination of intracellular compartmentalization and specific inhibitors of microtubule assembly. Compartmentalization of tubulin might occur by association of tubulin with membranes or lipid complexes within the egg (13, 30, 54) such that the free soluble tubulin concentration is $<0.36 \text{ mg/ml}$ (the critical concentration at 22°C). Weisenberg (68, 69) has reported that 10-20% of the tubulin in surf clam oocytes is particulate and is found associated with a granular and membranous material. Also, several inhibitors of microtubule assembly have been described and characterized in marine eggs as cytoplasmic RNA (9), RNA-inactivated inhibitors (72), proteases (3), and an inhibitor protein from sea urchin cortices (42). In addition, calcium was recently shown to be an effective regulator of unfertilized egg tubulin assembly (55) and mitotic spindle tubulin (27) assembly in vitro from the sea urchin *S. purpuratus*.

Surf clam meiotic spindle tubulin was nearly identical to the pool of cytoplasmic tubulin in unfertilized eggs when compared by isoelectric focusing, one-dimensional peptide mapping, and in vitro assembly studies. These data suggest that meiotic spindle tubulin does not become competent to assemble by a chemical modification of a subset of the total pool of tubulin in unfertilized surf clam oocytes. Further, these data suggest that the positive regulation of the in vivo assembly of spindle microtubules is dependent most likely upon the appearance or unmasking of mitotic organizing centers (23, 45, 46, 59) or the synthesis or release of assembly-promoting factors in the embryo, rather than changing the polymerization competency of the tubulin itself. However, while polymerization-competence is not altered by egg activation, it is still possible that important post-translational modifications of spindle tubulin such as tyrosinolation or rapidly reversible phosphorylation and dephosphorylation

could take place; modifications that would go undetected by our methods. Such possible modifications may be of importance to *in vivo* tubulin functions other than polymerization.

Conserved Properties of Oocyte and Embryo Tubulin

Surf clam oocyte and meiotic spindle tubulin both self-assembled into microtubules at low protein concentrations at physiological temperatures in the absence of assembly-promoting MAPs. MAP-free unfertilized sea urchin egg tubulin (57) and mitotic spindle tubulin from the same sea urchin species (26) also self-assembled at physiological temperatures (15–18°C) at low protein concentrations. Similarly, starfish and sand dollar egg tubulin isolated by DEAE chromatography (56) assembled in the absence of MAPs at protein concentrations as low as 0.5 mg/ml. These results differed strikingly from anion-exchange purified mammalian brain tubulin that, even at 37°C, only assembled at protein concentrations of 2–8 mg/ml (36, 39). Thus, these assembly properties of tubulins, isolated from phyla as evolutionarily divergent as echinoderms and molluscs, suggest that they are conserved properties of tubulin common to poikilotherms, oocytes, or rapidly dividing embryos.

Despite the conservation of protein sequence of the tubulin molecule during evolution, surf clam oocyte and meiotic spindle tubulin (25, 56), sea urchin egg and mitotic spindle tubulin (26, 27, 49, 57), and mammalian brain tubulin are unique tubulins. Since the tubulins are isolated from cells with potentially different microtubule functions, the differences observed *in vitro* among marine egg and oocyte tubulins and among egg tubulins and mammalian brain tubulins are likely to reflect differences in the functions of these tubulins *in vivo*.

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